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## **Thyroid disruption in zebrafish (*Danio rerio*) larvae: Different molecular response patterns lead to impaired eye development and visual functions**

Baumann, Lisa ; Ros, Albert ; Rehberger, Kristina ; Neuhauss, Stephan C F ; Segner, Helmut

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**Title:** Thyroid disruption in zebrafish (*Danio rerio*) larvae: different molecular response patterns lead to impaired eye development and visual functions

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The vertebrate thyroid system is important for multiple developmental processes, including eye development. Thus, its environmentally induced disruption may impact important fitness-related parameters like visual capacities and behaviour. The present study investigated the relation between molecular effects of thyroid disruption and morphological and physiological changes of eye development in zebrafish (*Danio rerio*). Two test compounds representing different molecular modes of thyroid disruption were used: propylthiouracil (PTU), which is an enzyme-inhibitor of thyroid hormone synthesis, and tetrabromobisphenol A (TBBPA), which interacts with the thyroid hormone receptors. Both chemicals significantly altered transcript levels of thyroid system-related genes (TR $\alpha$ , TR $\beta$ , TPO, TSH, DIO1, DIO2 and DIO3) in a compound-specific way. Despite these different molecular response patterns, both treatments resulted in similar pathological alterations of the eyes such as reduced size, RPE cell diameter and pigmentation, which were concentration-dependent. The morphological changes translated into impaired visual performance of the larvae: the optokinetic response was significantly and concentration-dependently decreased in both treatments, together with a significant increase of light preference of PTU-treated larvae. In addition, swimming activity was impacted. This study provides first evidence that different modes of molecular action of the thyroid disruptors can be associated with uniform apical responses. Furthermore, this study is the first to show that pathological eye development, as it can be induced by exposure to thyroid disruptors, indeed translates into impaired visual capacities of zebrafish early life stages.

**Keywords:** zebrafish, thyroid disruption, eye development, behaviour

**Abbreviations:** PTU, propylthiouracil; TBBPA, tetrabromobisphenol A; EDC, endocrine disrupting compound; MoA, mode of action; DMSO, dimethylsulfoxide; dpf, days post fertilization; TR, thyroid receptor; DIO, deiodinase; TPO, thyroperoxidase; TSH, thyroid-stimulating hormone; PFA, paraformaldehyde; OKR, optokinetic response; T3, triiodothyronine; T4, thyroxine; RPE, retinal pigment epithelium

## 1. Introduction

Environmental contaminants with endocrine disrupting properties (endocrine disrupting compounds, EDCs) affect wildlife and humans by interfering with endocrine homeostasis and hormonal processes, e.g. oestrogen or thyroid signalling. The possible adverse outcomes of EDC exposure have become a major issue in ecotoxicology and human toxicology, including the scientific challenges to understand the molecular and physiological modes of action (MoA) of EDCs as well as their ecological consequences. An important endocrine system that is targeted by a variety of environmental contaminants is the thyroid hormone system. Field surveys, epidemiological studies as well as laboratory experiments on birds (Brouwer et al., 1998), amphibians (Carr and Patiño, 2011), fish (Brar et al., 2010; Brown et al., 2004) or humans (Patrick, 2009) provide ample evidence that exposure to environmental pollutants can be associated with disruption of thyroid hormone synthesis, metabolism and / or transport and thus can lead to altered thyroid hormone status and thyroid hormone signalling. Thyroid-disrupting effects have been reported for, e.g. polychlorinated biphenyls, polyaromatic hydrocarbons, polybrominated diphenyl ethers, organochlorine pesticides as well as different metals (Brown et al., 2004; Yu et al., 2013). Those environmental chemicals appear to interfere with the thyroid system of vertebrates by two principal molecular mechanisms:

- a) Disrupting the thyroid hormone homeostasis, which includes effects on enzymes responsible for iodine uptake and thyroid hormone synthesis, and transport in the blood, on cellular uptake degradation and excretion = thyroid gland function disruptors (Thienpont et al., 2011).
- b) Ligand binding to the thyroid receptors in the peripheral target tissues and subsequent activation of thyroid signalling; this MoA does not necessarily involve changes in endogenous hormone levels but in thyroid signalling = thyroid hormone action disruptors (Boas et al., 2012).

Besides these basic molecular changes associated to thyroid disruptors, the physiological and fitness consequences of environmental exposure remain less investigated. It is known that the thyroid system plays an important role in key physiological processes such as growth, development and reproduction, therefore, its environmentally induced disruption may impact

a wide variety of health and fitness-related parameters such as larval survival, behaviour, fertility or metamorphosis (Power et al., 2001).

An important part of fish development is the differentiation of the eyes and craniofacial structures, as these are essential for predator avoidance and food finding/intake. Thus, malformations of the eyes and retinal cells might ultimately translate in a reduction of their individual fitness. The eye and craniofacial development of vertebrates is a complex process that is partially regulated by thyroid hormones (Bohnsack and Kahana, 2013; Darras et al., 2015). Knock-down of specific thyroid-regulating genes as well as treatment with different thyroid disruptors resulted in reduced eye size, malformations of eyes and head, and disruption of retinal pigment cell layers of zebrafish larvae (Heijlen et al., 2014; Reider and Connaughton, 2014). It has been reported that cone photoreceptor development seems to be under direct regulation of thyroid hormones (Raine and Hawryshyn, 2009; Suzuki et al., 2013), and it has been described, that thyroid receptors are expressed in the outer nuclear layer of the retina, which contains the developing photoreceptors (Bertrand et al., 2007). The exact pathways still need further investigation, even though a recent study could show that different genes involved in phototransduction are changed in their expression after knockdown of deiodinase enzymes in zebrafish, which regulate intracellular thyroid hormone levels (Bagci et al., 2015). However, many factors involved in the complex thyroidal regulation of eye development in fish remain unclear and need to be clarified and investigated. One particularly relevant but yet unanswered question is whether the phenotypic outcome (altered eye development) differs with the molecular MoA of the thyroid disruptors.

While the role of thyroid hormones in vertebrate eye development is well proven, and although a number of studies have shown that disruption of the thyroid hormone system affects eye morphology of developing fish, we lack clear proof that this translates into impaired functionality of the optic system. How does the disrupted eye development due to thyroid disruption result in direct physiological impairment of the developing fish? For understanding the impact of thyroid disruptors on fish we have to show which specific developmental processes are disrupted, but additionally it needs to be shown what the implications are for functional capacities of the organism. Thus, the central hypotheses to be tested in the present study are (i) that different molecular initiating events of thyroid disruption lead to the same dysfunctional eye development and morphology of developing zebrafish larvae and (ii) that the disruption of morphological eye development translates into functional impairment of visual performance of the exposed fish.

To verify or reject our hypotheses, we treated zebrafish embryos with two thyroid disrupting compounds that display different molecular actions on the thyroid system – inhibition of thyroid hormone synthesis with propylthiouracil (PTU) versus interaction with the thyroid receptors by tetrabromobisphenol A (TBBPA). Even though both substances seem to have multiple effects on the thyroid system, there is clear evidence that the basic MoAs differ, which is essential for our further investigations on disrupted eye development. PTU is a thiouracil-derived drug that is widely used to inhibit elevated thyroid hormone production in humans. It inhibits iodine and thyroperoxidase (TPO) from their normal interactions with thyroglobulin to form thyroid hormones. Thus, exposure to PTU results in lowered thyroid hormone levels (Van der Ven et al., 2006a). Consequently, the chemical often serves as reference substance in exposure experiments on the thyroid system. Recent studies revealed the adverse effect of PTU on the zebrafish thyroid system (Schmidt and Braunbeck, 2011) and embryonic development (Jomaa, 2014). Also effects of PTU on fish eye development have been reported (Macaulay et al., 2015; Raine and Hawryshyn, 2009), which makes it an interesting candidate for the current project. Due to its presence in the environment and biota (Environment Canada, 2013; Kitamura et al., 2002), TBBPA was chosen as exposure chemical in the present study as representative for a thyroid action disruptor. It is a brominated flame retardant and derivative of bisphenol A with different thyroid disrupting capacities. It is able to interact with the thyroid receptors either as agonist, or as antagonist, depending on the concentration and the endogenous TH levels of the organism (Crofton, 2008; Kitamura et al., 2002; Zhang et al., 2014). But also interaction with binding proteins and hepatic clearance were reported (Boas et al., 2012). Previous studies have shown that TBBPA competes with T4 in its binding to the plasma-transporter protein, transthyretin (Hamers, 2006). TBBPA is acutely toxic in high concentrations and disrupts development and reproduction of zebrafish at low concentrations (Kuiper et al., 2006). Moreover, it impairs thyroid hormone-dependent metamorphosis in amphibian models (Fini et al., 2007; Jagnytsch et al., 2006). In zebrafish changes in gene expression of thyroid-related genes have been reported (W. Chan and K. Chan, 2012).

## 2. Materials & Methods:

### 2.1. Test chemicals

Tetrabromobisphenol A (TBBPA, CAS 79-94-7) and Propylthiouracil (PTU, CAS 51-52-5) were purchased from Sigma Aldrich. Exposure concentrations were 0, 100, 200, 300 and 400 µg/L for TBBPA (resp. 0, 0.18, 0.37, 0.55, 0.73 µM) and 0, 50, 100 and 250 mg/L for PTU (resp. 0, 0.29, 0.59, 1.47 mM). These concentrations were chosen based on range-finding tests to determine treatments that were sub-lethal. For TBBPA we selected concentrations that were lower than 1.09 mg/L, the 96h median effective concentration for zebrafish embryo treatments (W. Chan and K. Chan, 2012). Moreover, the range of concentrations we used (100-400 µg/L for TBBPA) have been shown to be well supported for larvae up to 8 days post fertilization, but showing clearly observable malformations at 400 µg/L (Wu et al., 2015). Treatment concentrations of PTU have been reported to result in depigmentation and clear effects on the thyroid at levels above 50 mg/L (Schmidt and Braunbeck, 2011; Van der Ven et al., 2006b). TBBPA was dissolved in DMSO with a maximal final concentration in the exposure medium of 0.04%. Both chemicals were applied in E3 embryo medium (zfin.org, 5 mM NaCl, 0.17 mM KCl, 0.33 mM CaCl<sub>2</sub>, 0.33 mM MgSO<sub>4</sub>).

### 2.2. Zebrafish maintenance and exposure

A breeding group of 250 adult wildtype (AB strain) zebrafish (*Danio rerio*) was kept in a 210 L recirculating mass-spawning breeding tank for egg production (Fleuren&Nooijen, Netherlands). Water parameters were constantly held at 27°C, pH 7.8 - 8.0 and day/night cycle of 14/10 hours. Freshly fertilized eggs were collected in the morning, washed in E3 medium and then distributed into pre-incubated (= plates were filled with the solutions 1 day before start of the test. For the start of the test, solutions were renewed) 24-well plates containing the exposure solutions latest 2 hours after fertilization (1 egg per well, 2 ml solution per well). Plates were incubated at 28°C under a day/night cycle of 14/10 hours. Exposure solutions were renewed daily with freshly prepared solutions. The exposure experiments were stopped at 5 days post-fertilization (dpf) by euthanizing the larvae in 300 mg/L MS-222 (Sigma). The described set-up applies for all further experiments and was performed in at least 3 independent experiments (replicates). For measuring the effect on mRNA expression we carried out 6 replicates for TBBPA, and 5 replicates for PTU. For

measuring the effect on OKR we carried out 3 replicates for each chemical. For light-dark-preference we carried out 3 replicates for PTU and 4 for TBBPA. For morphology and histology 3 replicates each.

### 2.3. qRT-PCR

24 euthanized larvae (5 dpf) of one exposure plate were quickly frozen in a 2 ml reaction tube on dry ice and stored at -80°C for further processing. To isolate RNA a stainless steel bead (5 mm diameter, Qiagen) and 1 ml TRI Reagent solution (Sigma) were added to each tube, and larvae were homogenized in a Tissue Lyser (Qiagen) at highest frequency for 2 min. Subsequently, total RNA was isolated by the acid guanidinium thiocyanate-phenol-chloroform extraction according to the manufacturer's instructions. RNA concentration and quality was checked with a spectrophotometer NanoDrop (ND1000 Thermo Scientific). Isolation typically resulted in about 20 µl of 900 ng/µl RNA with 260nm/280nm ratio ranging between 2.0 and 2.2. When the purity ratio (260nm/230nm) was lower than 1.8, LiCl was added, the precipitate was restituted with pure water, and the concentration and purity was measured again using the NanoDrop. Afterwards DNase digestion and cDNA synthesis with 1 µg RNA per reaction were performed with Promega GoScript™ Reverse Transcriptase, as described in the instructions.

qRT-PCR was run using a standardized protocol based on Promega GoTaq® qPCR Master Mix which uses Broyt™ Green dye to detect DNA formed during the PCR amplification. 96-well fast qPCR Plates (ThermoFisher AB-1900) were loaded with 7.5 µl Master Mix, 1.0 µl of one of the forward and reverse primers (Microsynth, 20 times diluted), 1.0 µl H<sub>2</sub>O and 1.5 µl of a cDNA sample. Plates were set-up to contain for each cDNA samples: 1) the primers for the reference gene in triplicates; 2) all of the target gene primers in triplicates, 3) a blank consisting of 1.5 µl H<sub>2</sub>O instead of cDNA to check for possible contaminations in duplicates. The qPCR was run on an Applied Biosystems 7500 Fast Real-Time PCR System, allowing a 5 min. period at 95 °C to denature the anti-Taq DNA polymerase antibodies in the GoTaq® qPCR Master Mix. The qRT-PCR amplification was then allowed to run for 40 cycles (3 sec. 95 °C / 30 sec. 60 °C). A dissociation phase (melting curve) was added to check for non-specific binding or multiple binding with the primers. Ct values were extracted using standardized settings: baseline was set to the average of normalized Rn values at cycle 3 to 7, and the log Ct values were extracted from the resulting ΔRn values at a threshold value of 0.2



(Applied Biosystems Detection Software version 1.3.1). 18S was used as reference gene as it gave stable Ct values over treatments. Other potential reference genes *elfa*, *Ribo11*, *G6P*, and *TATA* were down-regulated in the higher treatment groups and thus not used (supplemental data). Efficiency of target and reference gene primers were compared and showed good linear relationship ( $r^2 > 0.9$ ). Fold induction was calculated using the  $2^{-\Delta\Delta Ct}$  method.

The expression of thyroid receptor alpha and beta ( $TR\alpha$  and  $TR\beta$ ), thyroperoxidase (TPO), thyroid-stimulating hormone (TSH) as well as, deiodinase type 1, 2 and 3 (DIO1, DIO2 and DIO3) were analysed using the following primers (5'-3' direction) (Q. Chen et al., 2012; Cheng et al., 2015; Pinto et al., 2013):

Tab. 1: list of primers used for qRT-PCR (synthesized by Microsynth Balgach, Switzerland)

Gene	Sequences of forward (fwd) and reverse (rev) primers
TR $\alpha$	fwd: 5'-CTATGAACAGCACATCCGACAAGAG rev: 5'-CACACCACACACGGCTCATC
TR $\beta$	fwd: 5'-TGGGAGATGATACGGGTTGT rev: 5'-ATAGGTGCCGATCCAATGTC
TPO	fwd: 5'-CCAGCCAGACCTCGTTC rev: 5'-CGGAGATGAGCGGAAGAAG
TSH	fwd: 5'-GCAGATCCTCACTTCACCTACC rev: 5'-GCACAGGTTTGGAGCATCTCA
DIO1	fwd: 5'-GTTCAAACAGCTTGTCAAGGACT rev: 5'-AGCAAGCCTCTCCTCCAAGTT
DIO2	fwd: 5'-GCATAGGCAGTCGCTCATTT rev: 5'-TGTGGTCTCTCATCCAACCA
DIO3	fwd: 5'- GAGACCGCTGATCCTCAACTTC rev: 5'- TCGATGTACACCAGCAGAGAGT
18S	fwd: 5'-CACTTGTCCCTCTAAGAAGTTGCA rev: 5'-GGTTGATTCCGATAACGAACGA

## 2.4. Size measurements and histology

For measurements of total body length and eye size, 24 euthanized larvae (5 dpf) from one exposure plate were transferred into 4 % PFA and stored at 4 °C. 10-11 larvae per treatment (from 3 independent replicates respectively) were measured using a stereomicroscope (Nikon SMZ745T) with camera (the imaging source, dfk72buc02), using NIS elements software to estimate the sizes with  $\mu\text{m}$  precision. Total length was measured as the length of the line between mouth and tip of the tail. When larvae were curved correction for this by drawing a path through the middle of the body was done. Eye size was measured at the longest diameter of the eye. Relative eye length was calculated as the ratio of eye length over total length expressed as a percentage.

For histological analysis of the eyes 24 euthanized larvae (5 dpf) from one exposure plate were transferred into 4 % PFA and fixed over night at 4 °C (3 independent replicates respectively). Subsequently, they were transferred into an agarose mold as described before (Sabaliauskas et al., 2006) and aligned in the same orientation. The agarose block containing the larvae was then processed in an embedding machine (Medite TPC 15Trio) in an ascending series of ethanol, followed by xylol and finally paraffin. 3  $\mu\text{m}$  thick paraffin slides (coronal sections) were produced with a rotating microtome (Microm HM340E). Slides were stained with haematoxylin/eosin (using a Leica ST5020 and CV5030) and then analysed under a light microscope (Olympus BX51) with camera (Olympus DP27). The associated software (CellSense, Olympus) was used to analyse the taken pictures by measuring the diameter of the different retinal cell layers of the larvae. If possible, at least 3 slides of each of the 2 eyes of each zebrafish larva were analysed. The cell diameter was measured at 8 different locations in the retina. Moreover, the pigmentation of the pigment cell layer was quantified at these 8 locations by measuring the grey values (0 = black, 255 = white).

## 2.5. Optokinetic response (OKR)

The OKR, a compensatory ocular motor reflex triggered by large scale movements in the surround, was assessed as described before (Huber-Reggi et al., 2014). In summary, single larvae (5 dpf) were taken from the exposure plates (24-well plate, 1 larva per well in 2 ml) and placed dorsal-up in a 35 mm Petridish containing 3 % pre-warmed (28°C) methylcellulose prepared in E3 medium. We carried out 3 independent replicates on different days. The larva was placed in the centre of a paper drum on which a computer-generated

visual stimulus (vertical black-and-white stripes rotating around the fish) was projected via a wide-angle conversion lens and a mirror from a LCD projector (PLV-Z3000; Sanyo). Computer-generated gratings stimulated the larva binocularly and the eye position was automatically tracked over time. The response of the eyes to different contrasts, velocities and frequencies of the moving stripes was assessed by recording of an infrared-sensitive CCD camera (Guppy F-038B NIR; Allied Vision Technologies). The term contrast describes the distinction between black and white moving stripes, which changed in the range of 0-100%. The term angular velocity is defined as the speed in which the stripes were rotating around the larvae (degrees per second). Spatial frequency describes the frequency of direction changes of the stripes and is defined as cycles per degree. The resulting eye velocity (degree per second) was calculated in real time. Data were analysed by custom-developed software written in MATLAB (MathWorks).

## 2.6. Swimming activity and light-dark preference

In addition to measuring the OKR, it was measured whether changes in light would result in changes in phototactic swimming activity (X. Chen and Engert, 2014). To do so larvae (5 dpf) were tested in their treatment plates. In total for PTU 3 independent experiments were carried out (275 larvae), and for TBBPA 4 replicates were performed (449 larvae). The 24-well plate (1 larva per well in 2 ml solution) was placed on top of a horizontally mounted LCD screen (Fujitsu Scenicview, brightness:  $0.03 \text{ cd/cm}^2$  or about 5000 lux per well [ $1.9 \text{ cm}^2$  3 mm distance]), and under each well a half-light circle (see fig. 1) was projected using a PowerPoint presentation (Microsoft). The circles were inverted after 2 minutes and this was repeated 3 times, resulting in 4 periods (for example: left dark – right dark – left dark – right dark). A camera (Nikon D90) was mounted one meter above the screen and from this point every 30 seconds a picture was taken. Ambient light was blocked by placing a black paper shield around the screen and camera. From a total of 16 pictures the following parameters were taken: (i) background preference: did the larva prefer the light or dark part of the well. For background preference only the data of the second minute (3<sup>rd</sup> and 4<sup>th</sup> picture) per period was analysed to allow the larva to swim to the preferred part in the well; (ii) general movement: a change in position from one to the other picture. This latter measurement was taken to test for possible changes of general activity due to impaired vision.

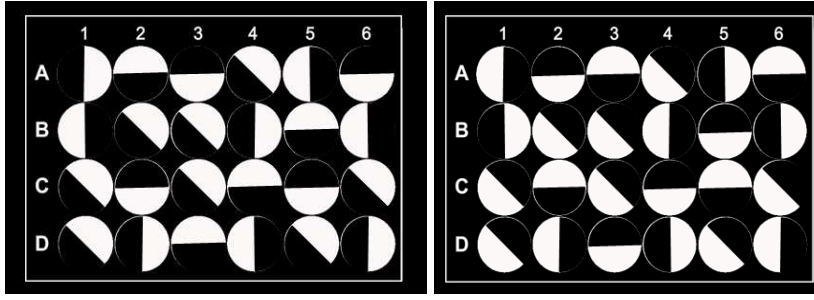


Figure 1: Projection of shades under the 24-well plate for behavioural observations.

## 2.7. Statistical analyses and graphs

Statistical tests were calculated using R (version 3.3.2). One-way ANOVAs with replicates were calculated using the lme4 package. Replicates were corrected in the error term of the model, degrees of freedom were estimated with Satterthwaite, and p-values were calculated using the lmerTest package. ANOVAs on independent datasets (histology) were calculated using lm (stats package). Deviations from normality in distributions of behaviour, mRNA expression, and histology were checked by Kolmogorov-Smirnov tests (nortest package) on the residual data of the ANOVA tests. These normality tests were non-significant (genes, behaviour, eye measurements) except for the CT values of TR $\alpha$  and TPO ( $p < 0.05$ ). Therefore, values for these genes were rankit transformed to calculate ANOVA statistics, after which the normality tests were non-significant. In order to test whether treatment doses of PTU and TBBPA were different from the control treatment we used the results of simple contrasts from the ANOVA. For OKR, statistical analysis was performed with SPSS Statistics 19 (IBM). OKR data were normally distributed (Kolmogorov-Smirnov test). A generalised linear model (GLM) was carried out on the eye velocity data of each eye to test for main effects of the OKR factors and the different treatments. Post-hoc Duncan tests were carried out for analysing which treatments were different from the control treatment.

A post-hoc analysis was added to check for possible relationships between molecular, morphological and behavioural data. In order to express these relationships each variable was summarized per treatment level and Pearson correlation coefficients were calculated. This analysis is supportive for some level of association between morphological eye measurements and behavioural measurements, but also shows clear differences regarding to treatment in the associations between these measurements and molecular changes. Since this analysis is very limited in n-value and only correlational we do not discuss the associations any further

because in order to test the causality of such relationships experimental manipulations of the variables are necessary, which was not the scope of this manuscript. A correlation matrix summarizing the results is provided as supplemental data.

All statistical analyses were two-tailed with the level of significance ( $\alpha$ ) set at 0.05. Graphs were made with R, using the graphics package.

### 3. Results

#### 3.1. Exposure to thyroid disruptors results in compound-specific alterations of transcript levels of thyroid-related genes

The gene expression pattern for the thyroid receptors alpha and beta differed clearly between both treatments. PTU caused significant down-regulation of mRNA expression levels of both thyroid receptors (see fig. 2B: TR $\alpha$ ,  $F[3,11.7] = 8.25$ ,  $p = 0.0032$ , TR $\beta$  only 250 mg/L,  $F[3,13.8] = 6.75$ ,  $p = 0.0049$ ). TBBPA instead, only had significant up-regulating effects on the expression of thyroid receptor alpha at low exposure concentrations (see fig. 2A: TR $\alpha$ ,  $F[4,23.1] = 5.71$ ,  $p = 0.0024$ ; TR $\beta$ ,  $F[4,23.1] = 2.36$ ,  $p = 0.083$ ).

Transcript levels of deiodinase type 1, 2 and 3 also differed between the treatments. PTU significantly up-regulated DIO2 ( $F[3,17] = 3.77$ ,  $p = 0.031$ ) and down-regulated DIO3 ( $F[3,9] = 7.60$ ,  $p = 0.008$ ), but did not affect DIO1 ( $F[3,12] = 1.53$ ,  $p = 0.26$ ); while TBBPA only slightly up-regulated DIO1 ( $F[4,23.0] = 3.09$ ,  $p = 0.036$ ), and had no significant on the other DIO types (DIO2:  $F[4,23.1] = 1.19$ ,  $p = 0.34$ ; DIO3:  $F[4,12] = 1.83$ ,  $p = 0.19$ ).

The treatment with both thyroid disruptors had no significant effect on transcripts of thyroid-stimulating hormone TSH (PTU:  $F[3,12.8] = 2.17$ ,  $p = 0.14$ ; TBBPA:  $F[4,22.3] = 0.46$ ,  $p = 0.76$ ), even though both seemed to up-regulate it.

Transcripts of the enzyme thyroperoxidase TPO were significantly changed in both treatments, but in the opposite direction. PTU strongly up-regulated TPO transcripts ( $F[3,12] = 6.68$ ,  $p = 0.0067$ ), while TBBPA down-regulated them ( $F[4,12] = 4.11$ ,  $p = 0.025$ ).

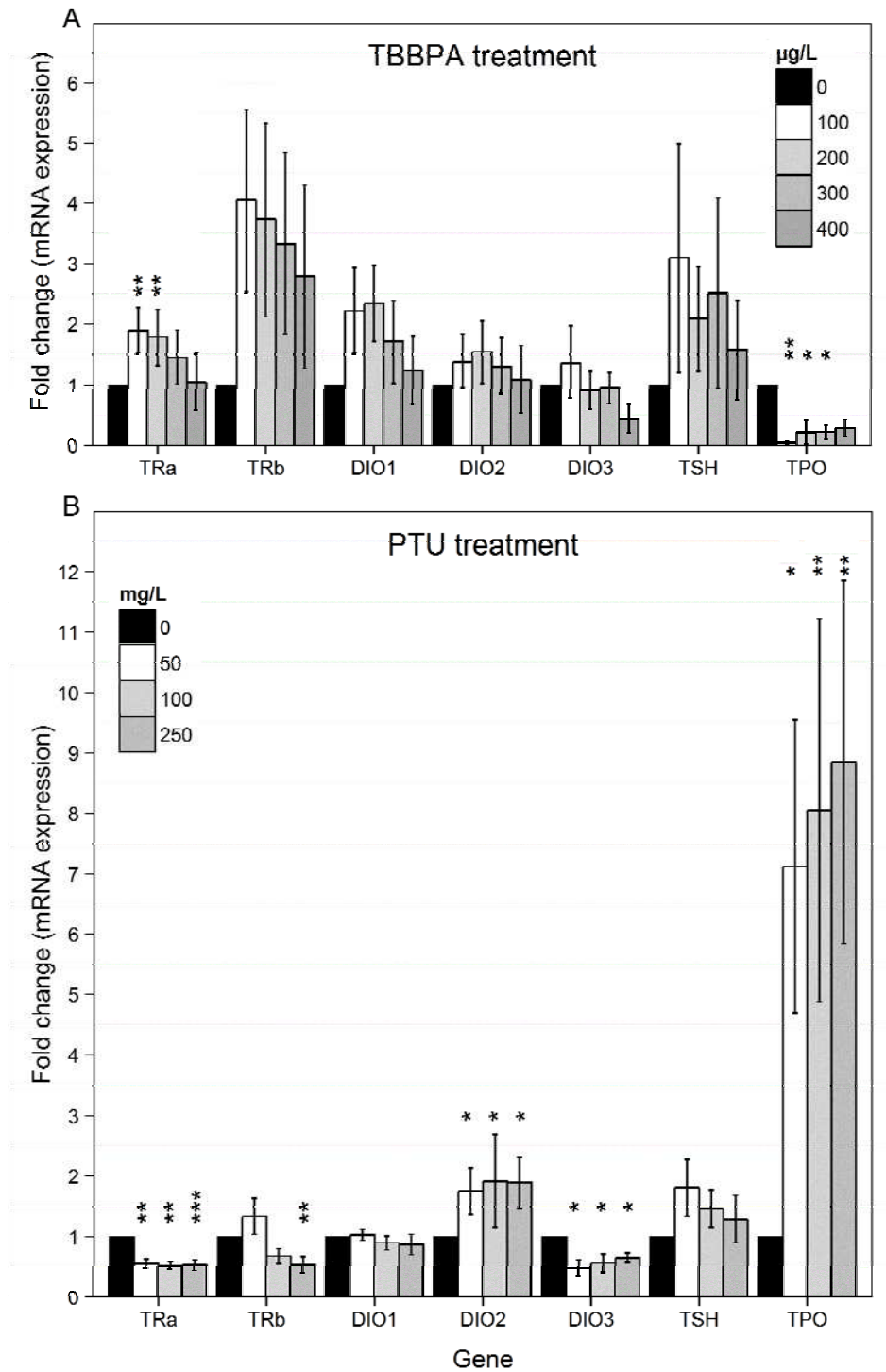


Figure 2: Changes in gene expression levels. TRa/b = Thyroid receptor alpha or beta; DIO1/2/3 = mRNA expression of deiodinase type 1, 2 or 3; TSH = thyroid-stimulating hormone; TPO = thyroperoxidase. Data are expressed as mean  $\pm$  st.err. Legend: bars with different colours represent different treatments. A: TBBPA treatment  $\mu\text{g/L}$ ; B: PTU treatment  $\text{mg/L}$ . Symbols represent differences of the simple contrast (ANOVA) between the control



group (concentration = 0) and the treatment groups: \* =  $p < 0.05$ , \*\* =  $p < 0.01$ , \*\*\* =  $p < 0.001$ .

### 3.2. Thyroid disruptor treatment of zebrafish larvae changes eye morphology and pigmentation

Both treatments had adverse effects on the development of the retinal pigment epithelium (RPE) layer in the eye of exposed larvae (see fig. 4). Histological analyses of coronal sections revealed a strong and significant concentration-dependent decrease in size and pigmentation of the retinal cells for PTU-treated fish (see fig. 3; ANOVA: size,  $F[3,35] = 20.76$ ,  $p < 0.001$ ; pigmentation,  $F[3,35] = 185.2$ ,  $p < 0.001$ ). Control fish had average RPE cell diameters of 7  $\mu\text{m}$ , which significantly decreased to 5  $\mu\text{m}$  in the highest exposure group (250 mg/L PTU), while these changes were statistically not significant for TBBPA-treated fish. Grey values (256-bit grayscale) increased from 25 (90% black) in the control to 60 (76% black) in the highest exposure group. Similar observations were made for TBBPA-treated fish, but clearly less pronounced than for PTU (ANOVA: size,  $F[3,50] = 3.33$ ,  $p = 0.027$ ; pigmentation,  $F[3,50] = 4.61$ ,  $p = 0.006$ ). Measurements of the other cell layers in the retina revealed no significant differences between control and treatments (data not shown).

Both total body length and eye size of exposed larvae (5 dpf) decreased due to TBBPA and PTU treatment (data not shown). To account for the changes in body morphology, eye size (EL) was corrected for body length (TL) (see fig. 5). The relative eye size was significantly decreased in both treatments (ANOVA EL/TL: TBBPA,  $F[3,28] = 8.47$ ,  $p < 0.001$ ; PTU,  $F[3,38] = 5.74$ ,  $p = 0.0024$ ).

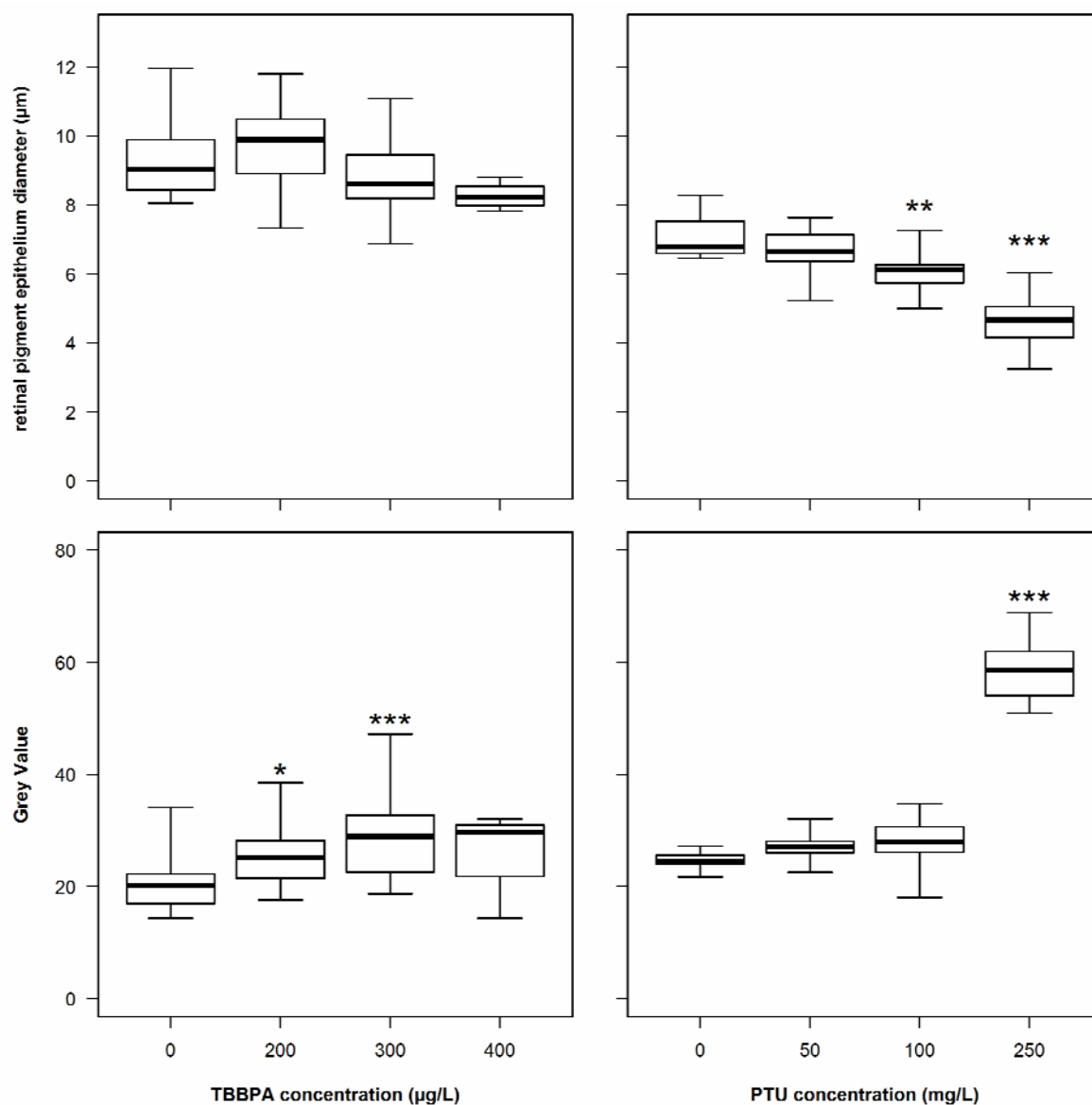


Figure 3: Cellular changes in the retina. The upper two panels depict the diameter of the retinal pigment epithelium. The lower two panels depict the grey value of the retinal pigment epithelium as measured from images using a 256-bit greyscale with 0 = black to 255 = white. For each individual 10 to 20 values were measured and averaged. Symbols represent differences of the simple contrast (ANOVA) between the control group (concentration = 0) and the treatment groups: \* =  $p < 0.05$ , \*\* =  $p < 0.01$ , \*\*\* =  $p < 0.001$ .

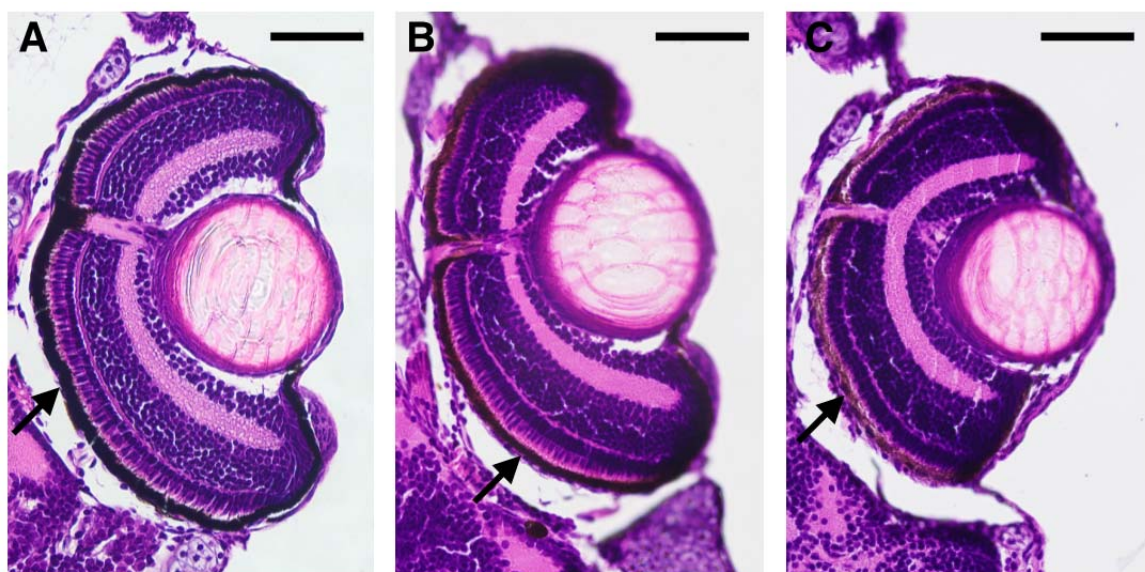


Figure 4: Morphological and cellular changes in the eyes of exposed zebrafish larvae (5 dpf). 3  $\mu$ m paraffin-sections, HE-staining. A: control, B: TBBPA (300  $\mu$ g/L), slight decrease of pigmentation C: PTU (250 mg/L), smaller eye size, less pigmentation. Arrows: retinal pigment epithelium. Scale bar: 100  $\mu$ m.

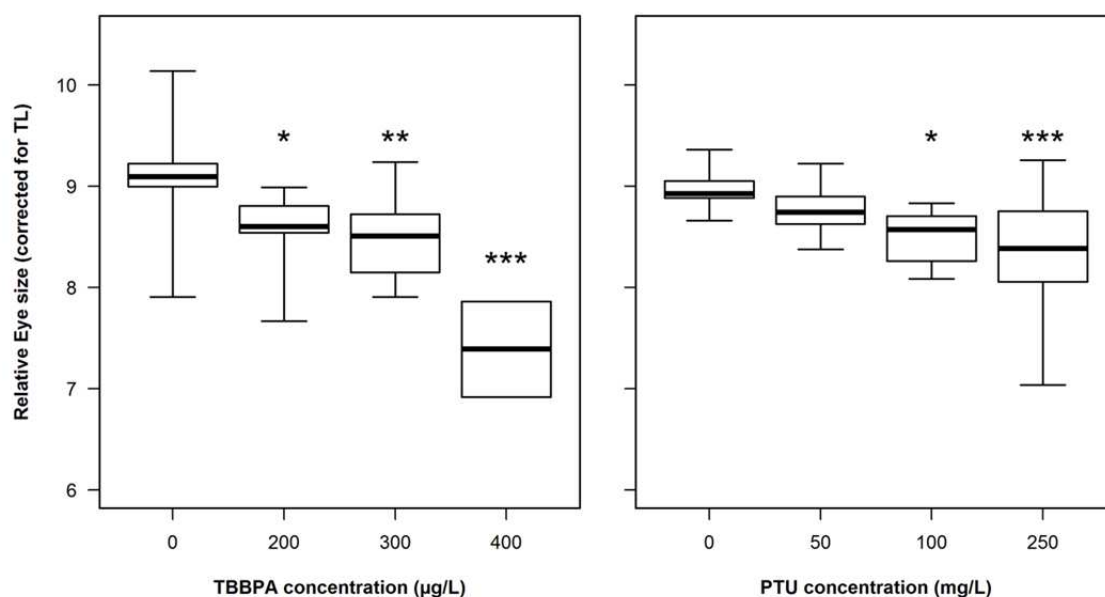


Figure 5: Eye size corrected for total body length of larvae ( $EL/TL \times 100\%$ ). Eye size was on average 0.13 mm and body length 1.4 mm. Symbols represent differences of the simple contrast (ANOVA) between the control group (concentration = 0) and the treatment groups: \* =  $p < 0.05$ , \*\* =  $p < 0.01$ , \*\*\* =  $p < 0.001$ .

### 3.3. Thyroid disruptor-induced eye malformations result in altered optokinetic response (OKR)

The visual performance of exposed larvae (5 dpf), as estimated by means of the OKR, decreased significantly with increasing concentration of TBBPA (ANOVA treatment effect: contrast,  $F[15,140] = 5.10$ ,  $p < 0.001$ , angular velocity,  $F[15,140] = 4.18$ ,  $p < 0.001$ , spatial frequency,  $F[15,140] = 4.47$ ,  $p < 0.001$ ). While larvae exposed to the lowest concentration of TBBPA (100  $\mu\text{g/L}$ ) showed no impairment of response to different contrast stimuli, those at 300 and 400  $\mu\text{g/L}$  were clearly not able to follow the visual signals with their eyes, which was statistically significant.

PTU-treated fish also showed clear impairment of OKR response in a concentration-depended manner (ANOVA treatment effect: contrast,  $F[15,140] = 16.75$ ,  $p < 0.001$ , angular velocity,  $F[15,140] = 7.85$ ,  $p < 0.001$ , spatial frequency,  $F[15,140] = 5.58$ ,  $p < 0.001$ ). While larvae exposed to 50 and 100 mg/L PTU only showed little deficiency in their OKR, larvae of the highest concentration (250 mg/L) were significantly impaired (see fig. 6).

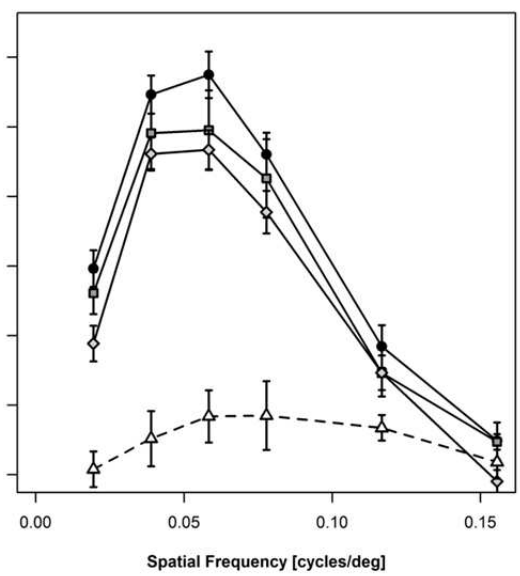
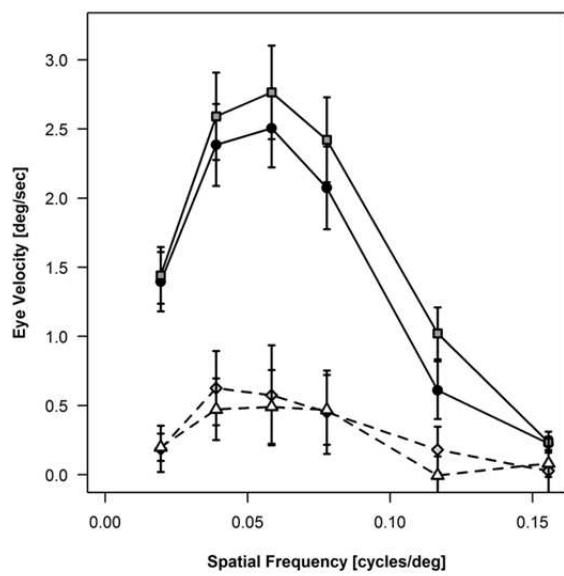
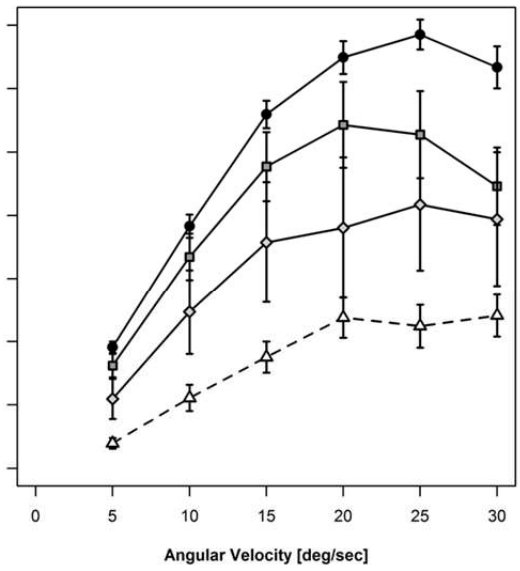
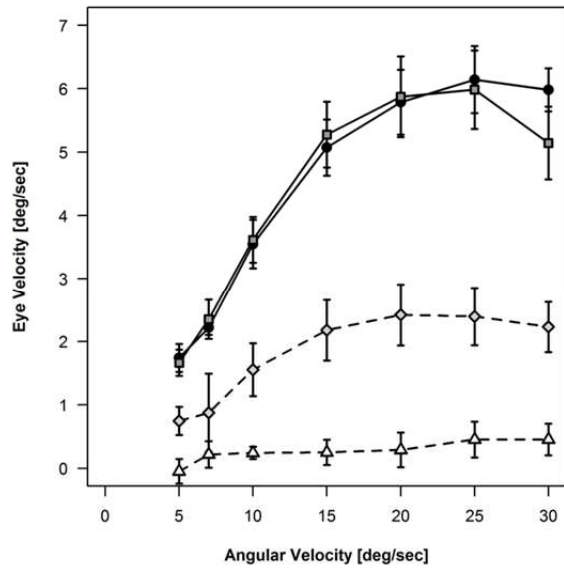
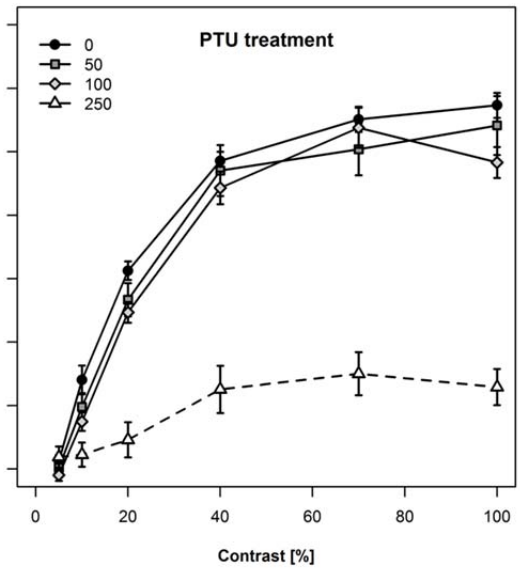
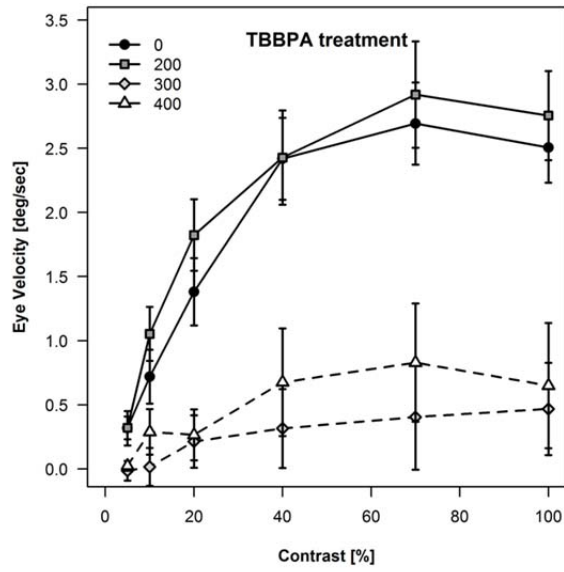


Figure 6: Changes in optokinetic response. Contrast (%) = distinction between black and white moving stripes; Angular Velocity (deg/sec) = speed in which the stripes were rotating around the larvae in degrees per second; Spatial Frequency (cycles/deg) = frequency of direction changes of moving stripes defined as cycles per degree. Data are expressed as mean  $\pm$  st.err. Legend: lines with different symbols represent different treatments. TBBPA concentrations are  $\mu\text{g/L}$  and PTU are  $\text{mg/L}$ . The groups with dotted lines differ significantly ( $p < 0.05$ ) from the control group.

### 3.4. Thyroid disruption alters swimming activity and light-dark background preferences

Both TBBPA and PTU had a significant effect on swimming activity of 5 days old larvae (ANOVA: TBBPA,  $F[4,16] = 19.47$ ,  $p < 0.001$ ; PTU,  $F[3,12] = 3.73$ ,  $p = 0.042$ ). However, both treatments did not result in a strong impairment of activity as only at the highest TBBPA and PTU concentrations activity was decreased less than two-fold. In fact, the effect of TBBPA on swimming activity was even stimulating at low TBBPA concentrations (see fig. 7, at  $300 \mu\text{g/L}$ ,  $p < 0.05$ ), and then significantly decreased at the highest exposure concentration (see fig. 7, at  $400 \mu\text{g/L}$ ,  $p < 0.001$ ). For PTU a suppressive effect on swimming activity was found, which was significant for treatment levels of 100 and 250  $\text{mg/L}$  (see fig. 7).

Control animals did not show any obvious background preference for the illuminated light half of the well over the dark half of the well (mean preference was 48.9 %). TBBPA did not change this preference (ANOVA:  $F[4,16] = 1.39$ ,  $p = 0.28$ ). However, PTU treatment resulted in a significant taxis towards the light half of the well (ANOVA:  $F[3,12] = 4.15$ ,  $p = 0.031$ ).

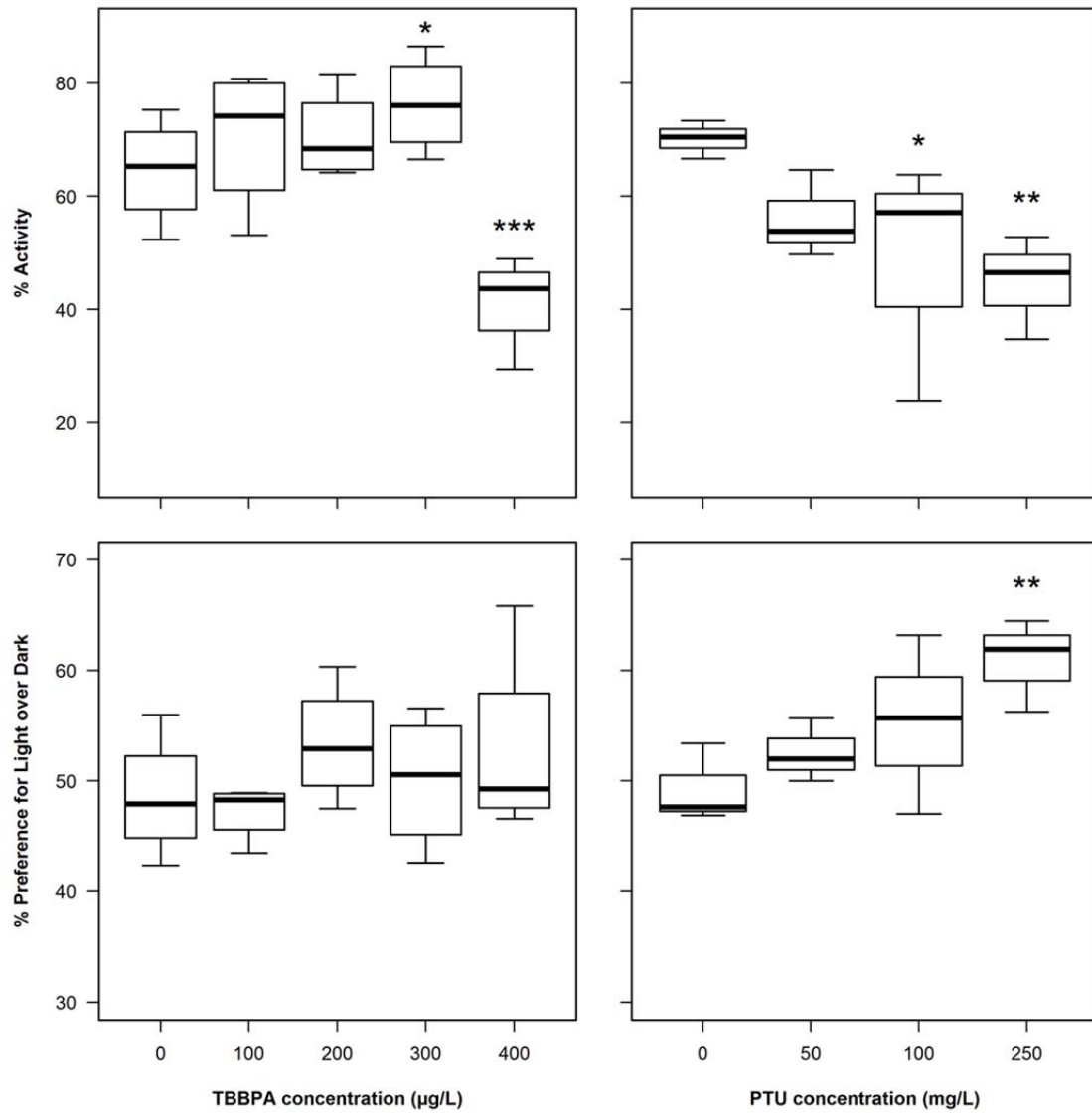


Figure 7: Swimming activity and light-dark preference. Symbols represent differences of the simple contrast (ANOVA) between the control group (concentration = 0) and the treatment groups: \* =  $p < 0.05$ , \*\* =  $p < 0.01$ , \*\*\* =  $p < 0.001$ .

## 4. Discussion

The thyroid hormone system is known to be important for eye development of vertebrates (Darras et al., 2015). Despite this knowledge, it remains unclear whether different molecular changes of the thyroid axis can translate into the same disruption of eye development. Moreover, the functional outcomes for the organism have not been clearly described yet. We aimed to address these open questions by comparing the impact of two different thyroid disruptors on the eye development of zebrafish embryos. On the one hand we hypothesized that the disruption of eye morphology is a converging event that is independent of the molecular MoA of the thyroid disruptor. Therefore, we chose two different thyroid disruptors with known MoA: PTU as an inhibitor of thyroid hormone synthesis and TBBPA as disruptor of the thyroid receptors. On the other hand, we hypothesized that the chemically-induced dysfunctional eye development will result in disrupted visual physiology and vision-related behaviour of the individual. We were able to confirm our hypotheses by demonstrating that the induced molecular changes differed in our treatments, but that the morphological outcome was very similar, and that the impaired eye development in fact was related to impaired visual capacities.

The basic starting point of our study was to choose two well-described thyroid disruptors with differing MoAs. As a proof-of-principle, we investigated the effects of both compounds on expression of thyroid-related genes to demonstrate the influence on different key players of the complex thyroid system. Both substances had significant impact on gene expression levels of thyroid-related genes in exposed larvae, but the reaction pattern differed clearly, which shows that the molecular changes affected different pathways within the thyroid hormone system of the developing fish. We investigated the 3 different types of deiodinase enzymes, which are important for regulation of thyroid hormone levels. Deiodinase type 1 (DIO1) and type 2 (DIO2) increase and deiodinase type 3 (DIO3) decreases local intracellular levels of T3, the most important active thyroid hormone. TBBPA had an up-regulating effect on the expression of DIO1 transcripts while PTU up-regulated DIO2 and down-regulated DIO3. Both indicate that either the enzymes themselves or the thyroid hormone levels were disrupted by the treatment, which confirms that the chosen compounds act indeed as thyroid disruptors. This is also obvious from the transcript changes found for the enzyme thyroperoxidase (TPO), which was strongly and significantly up-regulated by PTU-treatment, probably as a compensatory mechanism against the substance-induced inhibition of the



enzyme that is responsible for synthesis of thyroid hormones. The opposite effect was observed for the TBBPA treatment. TSH expression levels were slightly up-regulated in both exposures, which was not significant. Transcript changes for the thyroid receptors alpha and beta clearly differed between both treatments: lower levels of TBBPA up-regulated the thyroid receptor genes while PTU down-regulated these genes. These findings confirm the current knowledge about the effects of both substances on the fish thyroid system (De Wit et al., 2008; Quesada-García et al., 2014) and show that they were disrupting the thyroid system in a different, partly even contrasting manner. This was the initial starting point for our further investigations about the eye morphology and physiology of treated larvae.

Even though the molecular response obviously differed between both compounds used in our study, the associated cellular changes in the retina of exposed larvae were very similar in their response pattern, but not regarding the threshold: while PTU had a strong impact on the development of the retinal pigment epithelium (decreased size and pigmentation), the effects of TBBPA were similar but not as pronounced. The impact of PTU on eye development, opsin expression and pigmentation in fish has been demonstrated previously (Gan and Novales Flamarique, 2010; Macaulay et al., 2015; Raine et al., 2010). Effects of the PTU-related compound phenylthiourea have also been reported (Bohnsack et al., 2011; Li et al., 2012), and these authors describe reduced eye size and cellular changes after treatment with this commonly used inhibitor of pigmentation in zebrafish larvae. The impact of TBBPA on eye development has not been demonstrated yet. Only adverse effects on general and cardiac development of zebrafish have been observed (Wu et al., 2015; Yang and K. Chan, 2015). In our study, analyses of body size and eye size and the resulting ratio revealed that both substances significantly decreased the relative eye size of the developing larvae.

The next evident step in our study was to demonstrate whether the morphological changes have impact on the visual performance and vision-based behavioural capacities of the exposed larvae. In fact, while several studies have demonstrated that thyroid disruptors have an adverse effect on eye morphology (e.g. Reider and Connaughton, 2014), it has not been clearly shown yet whether this implicates impaired vision. Visual performance represents a key factor for the survival of developing fish embryos and larvae: behaviour concerning predator avoidance and feeding success is highly dependent on an intact optical sense. Thus, the present study provides novel information by verifying that impaired eye development translates into impaired vision and behaviour. We used the OKR, swimming activity and light-preference as fitness-related endpoints to investigate how general and vision-related

behaviours were altered by the treatment with the two different thyroid disruptors. The reaction pattern of the OKR was very similar for both treatments, showing a significant concentration-dependent decrease of visual capacities of the larvae. Partly comparable observations have been made by Reider and Connaughton (2015) who treated zebrafish embryos with the thyroid disruptor methimazol and observed increased anxiety behaviour. To our knowledge, this is the only study that could demonstrate how thyroid disruption and the resulting aberration of the eyes leads to altered behaviour in zebrafish larvae. Additional to the impaired OKR, we also found changes in light seeking and swimming behaviour of thyroid disruptor-treated larvae, which indicates that not only visual performance itself, but also vision-related behaviour was disrupted. Thus, we are the first to provide evidence that altered eye morphology, as we observed under exposure to different thyroid disruptors, indeed implies altered visual capacity and vision-related behaviour.

## **5. Conclusion**

The results of the present study provide initial evidence that thyroid disruptors which induce different molecular changes in the thyroid system can lead to uniform alterations in eye development of zebrafish larvae. This suggests that different molecular modes of action eventually merge into identical morphological outcomes, a hypothesis which will have to be further tested in detail in future studies. A second major finding from this study is that the impaired eye development, as it arises under exposure to the thyroid-disrupting compounds, indeed implies an impaired visual performance, as evidenced from physiological and behavioral tests. These findings are especially relevant, as development and functionality of the eyes are essential for the survival of fish larvae in the environment.

**Competing interests:** We have no competing interests.

**Author's contributions:**

LB and AR are equally contributing authors. LB carried out parts of the molecular and histological lab work, participated in data analysis, carried out OKR experiments, designed the study and drafted the manuscript; AR carried out parts of the molecular lab work, participated in data analysis, carried out the behavioural experiments, carried out the statistical analyses and helped draft the manuscript; KR participated in molecular lab work and fish maintenance, helped draft the manuscript; SN coordinated parts of the study and helped draft the manuscript; HS conceived of the study, designed the study together with LB, coordinated the study and helped draft the manuscript.

All authors gave final approval for publication.

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